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Hepatic flavin-containing monooxygenase activity attenuated by cGMP-independent nitric oxide-mediated mRNA destabilization

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Abstract

To identify the novel mechanism by which nitric oxide (NO) suppresses flavin-containing monooxygenase (FMO) activity in endotoxemic rat livers, NO-overproducing conditions were induced in primary cultured rat hepatocytes by treatment with a mixture (LCM) of lipopolysaccharide and proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ), or by the addition of a pure NO donor, spermine–NONOate. mRNA levels of the major hepatic form, FMO1, decreased via a cGMP-independent destabilizing effect of NO rather than by decreased transcription. The decrease in the mRNA levels caused by LCM-induced inducible NO synthase (iNOS) was completely blocked by co-treatment with aminoguanidine, a selective iNOS inhibitor. Furthermore, spermine–NONOate, but not the cGMP analog, 8-bromo-cGMP, dose- and time-dependently attenuated FMO1 mRNA stability in actinomycin-D-pretreated cells, resulting in decreases in protein levels and biochemical activity. These results suggest that NO acts directly in a cGMP-independent mechanism by decreasing the half-life of FMO1 mRNA, thereby inducing impairment of FMO-related functions in endotoxemia.

Keywords: Nitric oxide; Flavin-containing monooxygenase; mRNA stability; Actinomycin-D; Endotoxemia

Flavin-containing monooxygenases (FMOs; EC 1.14.13.8), which belong to the hepatic drug-metabolizing enzyme family, play a major role in the detoxification of endogenous and exogenous compounds [1,2]. Generally, FMO activity decreases in inflammatory conditions, for example, in patients with chronic viral hepatitis or in animal models of endotoxin-induced sepsis [3–5]. However, the causative mechanisms underlying the loss of FMO activity are not well understood.

In a previous study, we reported for the first time that FMO activity is suppressed in lipopolysaccharide (LPS)-treated endotoxemic rat livers by an inflammatory nitric oxide (NO)-mediated decrease in FMO1 mRNA levels [5]. Therefore, pre- or post-transcriptional modification of FMO1 (a major isoform in rat liver) by NO was suggested. We also found that NO directly and reversibly inhibits the biochemical activity of the major isoform, FMO3, in human liver by an S-nitrosylation mechanism [6,7]. In addition to the effect of NO on the FMOs, endogenously released NO and exogenous treatment with NO donors inhibit the activity of hepatic cytochrome P450s by several pathways [8–10]. In particular,

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a mixture of LPS and inflammatory cytokines stimulates the expression of inducible NO synthase (iNOS) in hepatocytes, and NO is thus overproduced [11]. This decreases hepatic cytochrome P450 activities in vivo, either by suppressing the transcriptional pathways or by direct inhibition of microsomal cytochrome P450 activities [12,13].

Recently, data have been reported on the NO-mediated stabilization or destabilization of the mRNAs of constitutive and inducible genes [14–19]. Furthermore, mRNA-modifying effects of NO have been detected that involve both cGMP-dependent and cGMP-independent mechanisms. Until now, however, there have been no reports of NO-mediated destabilization of FMO mRNAs.

In this study, we examined the effects of endogenously induced NO and an exogenously administered pure NO donor (spermine–NONOate) on post-transcriptional modifications, particularly on FMO1 mRNA stability, as a causative mechanism for the suppression of FMO activity under endotoxemic conditions.

Materials and methods

Chemicals. Aminoguanidine (AG), lipopolysaccharide (LPS), spermine–NONOate, ranitidine (RA), thiobenzamide (TB), actinomycin-D (Act-D), cycloheximide, and monoclonal antibody directed against β-actin were purchased from the Sigma Chemical (St. Louis, MO, USA). A polyclonal antibody directed against FMO1 was obtained from Gentest (Woburn, MA, USA). Pro-inflammatory cytokines, including recombinant human tumor necrosis factor α (TNF-α), recombinant rat interferon γ (IFN-γ), and recombinant human interleukin 1β (IL-1β), were purchased from R&D Systems (Minneapolis, MN, USA). RA metabolites, including N-oxide, used as standards in high-performance liquid chromatography (HPLC) analysis, were kind gifts from Dr. Carol Jenkins of GlaxoWellcome (Research Triangle Park, NC, USA). All other chemicals or solvents were of the highest grade and purchased commercially.

Isolation and culture of rat hepatocytes. Male Sprague–Dawley rats (Animal Breeding Laboratory of Inha University, Inchon, Korea) with a body weight of 170–180 g were allowed free access to a laboratory diet and were maintained under a 12:12 h light:dark cycle. Rat hepatocytes were prepared from the livers of male Sprague–Dawley rats by in situ perfusion of the liver with 0.025% collagenase (type IV) and plated on collagen-precoated (15 μg/cm^2 collagen) culture dishes (60 or 100 mm diameter) at a density of about 1×10^6 or 6×10^6 cells per plate, respectively, in an almost continuous monolayer. These cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco/BRL-Life Technologies, MD, USA). Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

Treatment of primary cultured hepatocytes with LPS plus cytokine mixture or NO donor. Hepatocytes were treated with 20 μg/ml LPS plus a mixture of pro-inflammatory cytokines (LCM), including 5 ng/ml TNF-α, 100 μg/ml IFN-γ, and 1 ng/ml IL-1β, for various periods (4, 8, and 12 h). In another experiment, the cells were pre-exposed to LCM for 6 h, after which Act-D was added to the medium for the periods indicated (2, 4, 6, and 8 h). The cells were co-treated with AG (0.5 mM) and LCM to selectively block inducible NO synthase (iNOS). In addition to the induction of endogenous NO in cells, cells were exposed to spermine–NONOate (sper/NO) for various times (1–8 h) and concentrations (0.05–1.0 mM). To determine the effects of time (0–12 h at 1 mM) and concentration (0–1 mM for 8 h) on FMO1 protein expression, sper/NO was added to the cell culture medium as indicated. The cells were also treated with a membrane-permeable cGMP analog, 8-bromo-cGMP, at three different concentrations (0.1, 0.5, and 1.0 mM) for 8 h. Cells were also pre-treated for 12 h with cycloheximide (30 μM) just before the addition of sper/NO (1 mM) to the cells, to identify the effects of NO on protein stability.

Measurement of a stable NO metabolite. NO metabolite (nitrite, NO_2^-) is a stable product of NO and is a reliable indicator of NO production in cell culture. Thus, nitrite released by LCM treatment was measured in the medium using the Griess assay [25]. Briefly, an aliquot of the supernatant was mixed with an equal volume of Griess reagent (1.0% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine in 5% phosphoric acid), and measured spectrophotometrically at 540 nm using a PowerWave/340 ELISA reader (Bio-Tek Instruments, Winooski, VT).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNAs were extracted from rat hepatocytes using TRIzol reagent (Sigma, MO, USA), according to the manufacturer's protocol. The cultured hepatocytes were washed twice with PBS and scraped off after adding TRIzol reagent. After treatment of detergent and differential centrifugation, the isolated total RNA precipitate was washed with 70% ethanol. Total RNA was then eluted with deionized distilled water containing diethylpyrocarbonate. Total RNA (1.5 μg) was then reverse transcribed for 30 min at 42 °C in the presence of avian myeloblastosis virus reverse transcriptase and oligo(dT)-Adaptor Primer (Takara, Japan). The PCR primers used to amplify the 674-bp fragment of rat FMO1 cDNA were: sense primer, from nucleotide (nt) 1033 to nt 1052, 5′-GTTGAGGATGGCCAGGCATC-3′; and antisense primer, from nt 1686 to nt 1706, 5′-GTTGGGTGTCTCTGGACGTGG-3′ (GenBank Accession No. BC061567). The β-actin primers used to normalize the loading of total RNA were: sense primer, 5′-AGAAGAGC TATGAGCTGCCT-3′; and antisense primer, 5′-CTTCTGCATCCT GTCAGCGATGC-3′ (amplifying a 236-bp fragment). PCR amplifications were performed in reaction mixtures containing 4 μl of single-stranded cDNAs. Thirty cycles of denaturation, annealing, and elongation at 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 90 s, respectively, were performed. The PCR products were resolved electrophoretically on a 0.8% agarose gel (NuSieve 1:1; FMC Bioproducts, Rockland, ME) containing 1 μg/ml ethidium bromide and then visualized under a UV transilluminator. The relative amounts of PCR products were compared using the image analysis software Bio1D (ver. 97; Vilber Lourmet, France).

Western blotting. Confluent cells incubated in DMEM containing 10% fetal bovine serum were washed with PBS and lysed in 0.3 ml of sample buffer [100 mM Tris–acetate (pH 7.4), 100 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF)] as described by Chung and Buhler [20]. Total microsomal protein (20 μg) of each sample was separated by sodium dodecyl sulfate–polyacrylamide gel (8–16%) electrophoresis and transferred to polyvinylidene fluoride membranes. The blots were probed with a polyclonal antibody specific for liver FMO1 at a dilution of 1:2000, which was detected with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase at a dilution of 1:5000 (Pierce, Rockford, USA). The blots were re-probed with a polyclonal antibody specific for actin at a dilution of 1:1000 (Sigma), which was detected with a goat anti-mouse secondary antibody conjugated to horseradish peroxidase at a dilution of 1:2000 (Pierce). Immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) kit (Amersham–Pharmacia Biotech, Piscataway, NJ), according to the manufacturer's instructions. Band intensities were quantified with densitometry, using a Duoscan model T1200 scanner (AgfaGevaert, NV, Germany) and Bio1D image analysis software (Vilber Lourmet).

Determination of microsomal FMO activity. FMO activity was determined from the metabolic oxidation rates of two known FMO substrates, RA and TB [21–24]. Briefly, 0.5 mg aliquots of hepatocyte microsomes were added to a reaction mixture (1 ml) containing 50 mM

Tris–HCl (pH 8.4), 0.25 mM $NADP^+$, 2 mM glucose 6-phosphate, 0.5 U glucose 6-phosphate dehydrogenase, and 7 mM $MgCl_2$. After 3 min of preincubation at 37 °C, 1 mM thiobenzamide was added to start the reaction. The rate of TB S-oxidation was determined spectrophotometrically at 370 nm for 5 min. To measure RA N-oxidation, 0.5 mg aliquots of hepatocyte microsomes were added to reaction mixture (0.5 ml) containing 0.1 mM potassium phosphate buffer (pH 8.4), 0.15 mM NADPH, and 1 mM RA. After incubation for 1 h at 37 °C, the microsomal preparations were centrifuged for 30 min at 100,000*g* to separate the supernatants. Aliquots of the supernatants (20 μl) were then injected into an HPLC system equipped with a KR100-5C18 column (4.6 × 150 mm, 4 μm; Eka Chemicals AB, Bohus, Sweden). The mobile phase consisted of a serial gradient of 50 mM NaH_2PO_4 containing 10% and 70% acetonitrile with a flow rate of 0.8 ml/min and UV detection at 320 nm, as described by Kang et al. [23] and Chung et al. [24].

Statistical analysis of data. Data are presented as means ± standard deviation (SD) values. The significance of the differences was determined using a simple Student's *t* test for comparison with controls or Kruskal–Wallis test for concentration-dependency of the estimated FMO activities and expressions. Statistical significance was assumed at *p* values of <0.05, <0.01 or <0.001.

Results

FMO1 mRNA expression and stability in LCM-stimulated hepatocytes

Using RT-PCR, we examined the effects of the endogenous NO released by stimulation with an LPS plus cytokine mixture (LCM) on the steady-state expression of FMO1 mRNA and its stability in primary cultured rat hepatocytes. LCM time-dependently attenuated FMO1 mRNA levels to less than 50% of the control level 12 h after treatment ($p < 0.05$ or $p < 0.01$), but this decrease was completely blocked by co-treatment with AG (0.5 mM), an iNOS-selective inhibitor (Figs. 1A and B). As shown in Fig. 1C, however, the concentration of NO metabolite (NO_2^-) increased ($p < 0.05$ or $p < 0.001$), and this increase was also completely blocked by co-treatment with AG. The stability of FMO1 mRNA was compared at five different time points (0, 2, 4, 6, and 8 h) after treatment with Act-D (0.2 μg/ml) in hepatocytes pre-exposed to LCM for 6 h (Fig. 2B). Interestingly, FMO1 mRNA levels that had decreased after pretreatment with LCM did not change over time after treatment with Act-D, because the action of iNOS was blocked by Act-D as shown in Fig. 2C. These results indicate that FMO1 mRNA levels are decreased by NO, and that the decrease in mRNA induced by pre-treatment with LCM is stable under these conditions, with little release of NO (Fig. 2).

Effects of spermine–NONOate on FMO1 mRNA stability

The effects on FMO1 mRNA stability of a pure NO donor, spermine–NONOate (sper/NO), administered exogenously to hepatocytes, were examined at four

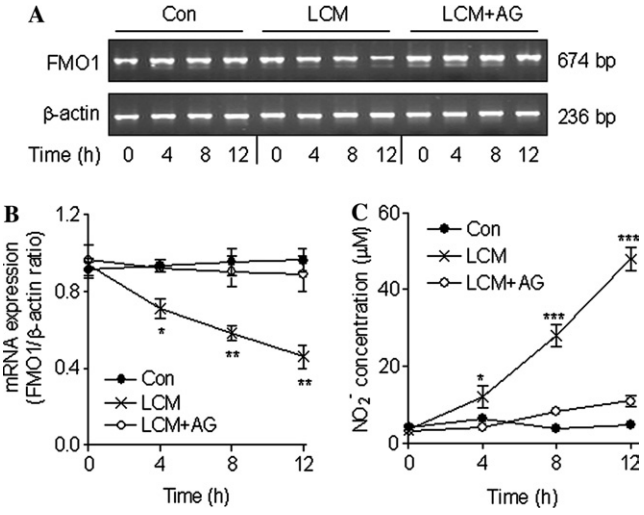

Fig. 1. Effects of LCM on levels of FMO1 mRNA expression and NO production in Act-D-untreated cells. Primary cultured rat hepatocytes were exposed to LCM or LCM plus AG at the concentrations described in Materials and methods. (A) FMO1 mRNA levels modulated by LCM or LCM plus AG pre-treatment were detected by RT-PCR. (B) In triplicate experiments, densitometric readings of FMO1 mRNA normalized to β-actin bands are expressed as the ratio of FMO1/β-actin mRNA. LCM treatment decreased time-dependently FMO1 mRNA levels (*$p < 0.05$ or **$p < 0.01$ vs controls, Student's *t* test at each time point), and the decreases were completely blocked in LCM plus AG-treated cells. (C) LCM treatment increased significantly NO release (NO_2^- concentration) at each time point (*$p < 0.05$ or ***$p < 0.001$ vs controls) and was blocked completely in cells co-treated with AG.

different time points (1, 2, 4, and 8 h) after co-treatment with Act-D (0.2 μg/ml). As shown in Fig. 3, sper/NO concentration- and time-dependently decreased FMO1 mRNA stability in Act-D-treated cells, whereas levels of FMO1 mRNA in the absence of the NO donor were constant, even after treatment with Act-D. Furthermore, the addition of sper/NO dose-dependently decreased the half-life of FMO1 mRNA: ~4.6 h at 0.05 mM, ~3.2 h at 0.1 mM, ~2.1 h at 0.5 mM, and ~1 h at 1 mM (Fig. 3B). These results clearly indicate that the destabilization of FMO1 mRNA is mediated directly by NO, and not by the action of the cGMP released by the NO-activated soluble guanylyl cyclase pathway, because soluble guanylyl cyclase expression was blocked. In addition, for semi-quantitative comparisons, NO-mediated decrease of FMO1 mRNA stability was re-evaluated by a manual cycle-dependent increase of PCR products at one time point (2 h) (Fig. 3C).

Effects of spermine–NONOate on FMO1 protein expression and stability

FMO1 protein levels were also investigated in the same cells shown in Fig. 3. As expected, FMO1 protein levels decreased markedly after treatment with sper/NO

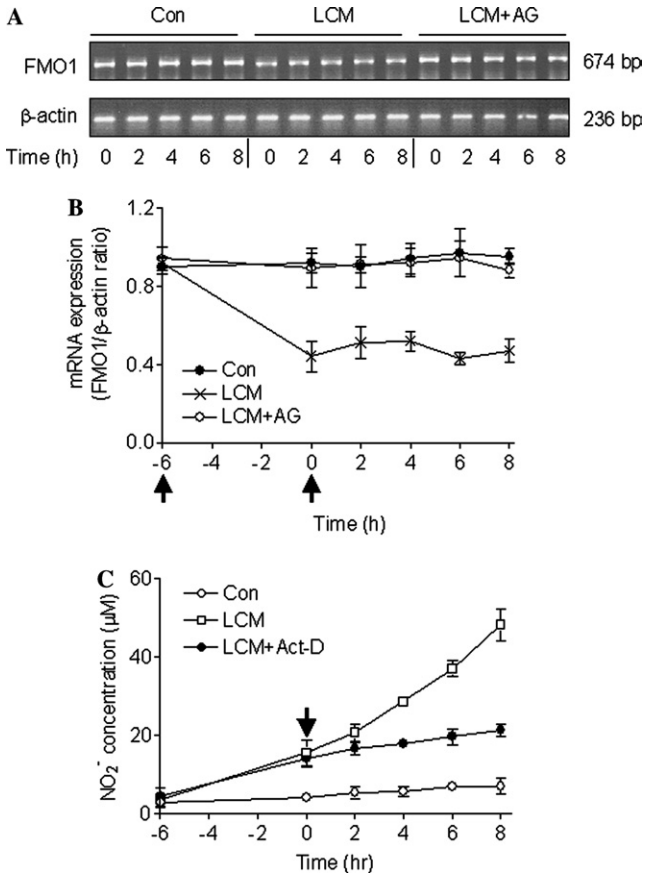

Fig. 2. Effect of LCM on levels of FMO1 mRNA in Act-D-untreated or -treated cells. (A) FMO1 mRNA levels modulated by LCM or LCM plus AG pre-treatment were detected by RT-PCR. (B) In triplicate experiments, densitometric readings of FMO1 mRNA normalized to β-actin bands are expressed as the ratio of FMO1/β-actin mRNA. Hepatocytes were pre-incubated with LCM or LCM plus AG for 6 h (left arrow) and then treated with Act-D (right arrow). (C) Under these conditions, the LCM-induced increasing rate of NO_2^- concentrations was markedly inhibited by treatment (arrow) of AG, suggesting the suppression of NO or NOS action.

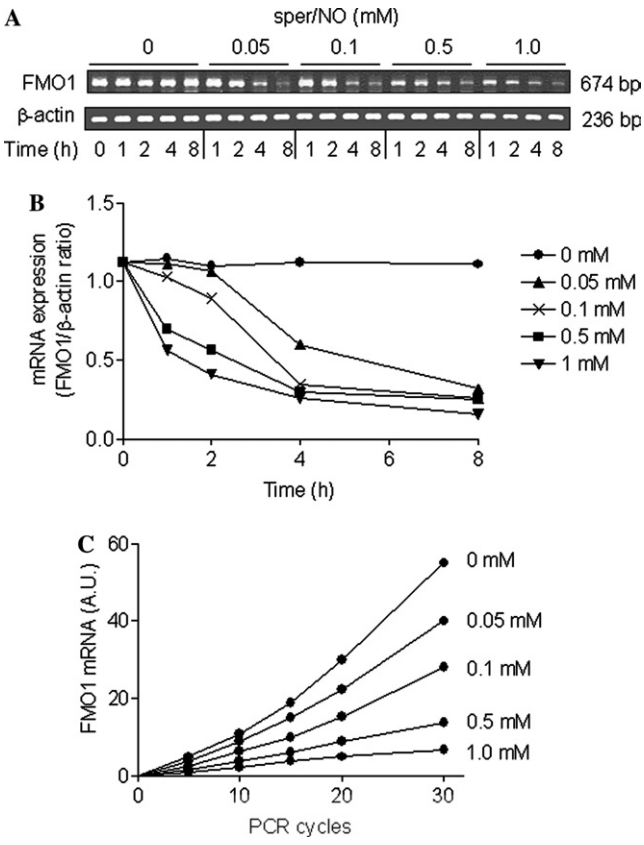

Fig. 3. Effect of sper/NO on the stability of FMO1 mRNA. (A) FMO1 stability was detected by RT–PCR in hepatocytes co-treated with sper/NO and Act-D for the times and at the concentrations indicated. (B) Densitometric readings of FMO1 mRNA normalized to β-actin bands are expressed as the ratio of FMO1/β-actin mRNA to compare the half-life of the mRNAs. (C) For semi-quantitative comparisons, NO-mediated decrease of FMO1 mRNA stability was also expressed as a graph demonstrating a manual cycle-dependent increase of PCR products at one time point (2 h).

when co-administered with Act-D (Fig. 4), in response to the direct or indirect NO-mediated degradation of FMO1 mRNA, as detected by RT-PCR. After cells had been co-treated with Act-D, FMO1 expression decreased time-dependently in sper/NO (1 mM)-treated cells, but not in untreated cells ($p < 0.05$ or $p < 0.01$ vs controls). The decrease in the levels of FMO1 protein was similar to the rate of destabilization of FMO1 mRNA ($p = 0.002$, Fig. 4). However, sper/NO did not affect FMO1 protein stability, as confirmed in cells co-treated for 12 h with the translation inhibitor, cycloheximide (30 μM) (data not shown).

Effects of spermine–NONOate on FMO activity

The effect of sper/NO on FMO activity was also examined in primary hepatocytes in the presence of Act-D. After co-treatment with sper/NO (0.05, 0.1, 0.5, or 1 mM) and Act-D (0.2 μg/ml) for 8 h, FMO activity was detected by HPLC and spectrophotometric analysis of the metabolic rates of two FMO substrates, ranitidine (RA) and thiobenzamide (TB). As shown in Table 1, FMO activity, determined by the rates of formation of RA N-oxide and TB S-oxide metabolites, was attenuated dose-dependently ($p = 0.007$ and $p = 0.011$, respectively, Kruskal–Wallis test).

Effects of cGMP analog on the expression of FMO1 mRNA and protein

Although the stability of FMO1 mRNA was mediated directly by NO without cGMP involvement, as shown in Fig. 3, we examined the effects of the cGMP analog, 8-Br-cGMP, on FMO1 mRNA stability. Sper/NO (1 mM) or membrane permeable 8-Br-cGMP (0.1, 0.5, or 1.0 mM) was administered for 8 h in the absence of Act-D, and the levels of FMO1 mRNA and protein expression were determined. Addition of

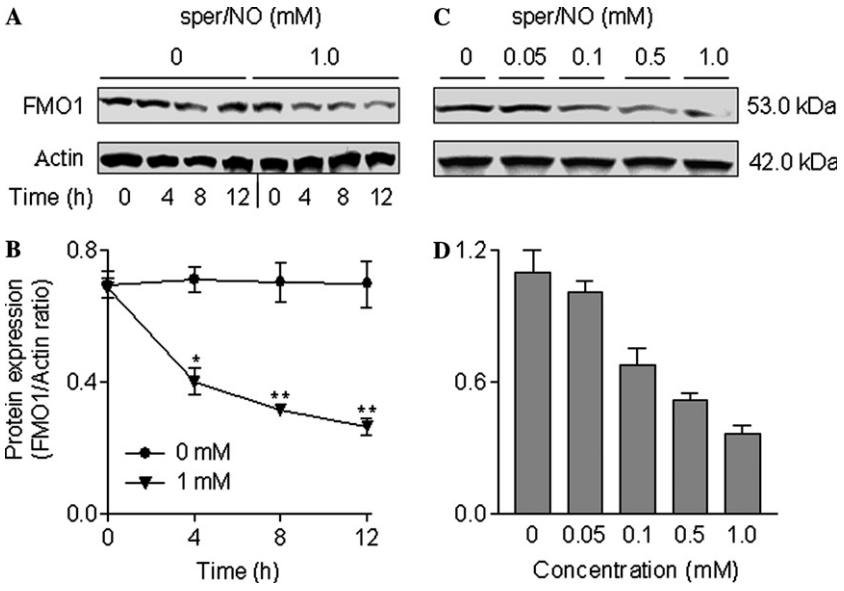

Fig. 4. Effects of sper/NO on levels of FMO1 protein expression in Act-D-treated cells. (A,B) In triplicate experiments, microsomal proteins isolated after Act-D treatment were resolved electrophoretically and detected with FMO1-specific antibody; and their expression levels are expressed as the ratio of FMO1/actin over time (*$p < 0.05$ or **$p < 0.01$ vs controls, Student's t test). (C,D) The protein levels decreased dose-dependently ($p = 0.002$, Kruskal–Wallis test).

Table 1
Effects of sper/NO treatment on FMO activity in the presence of Act-D

	Sper/NO (mM)				
	0	0.05	0.1	0.5	1.0
RA[a]	0.37 ± 0.02	0.32 ± 0.03	0.28 ± 0.01	0.21 ± 0.03	0.17 ± 0.01
TB[b]	12.27 ± 0.60	10.76 ± 0.89	9.83 ± 0.91	8.60 ± 0.51	7.51 ± 0.30

Results are expressed as means ± SD obtained from independent triplicate experiments for each concentration (nmol/min/mg protein). Dose-dependency was analyzed by Kruskal–Wallis test ([a]$p = 0.007$, [b]$p = 0.011$), RA, ranitidine; TB, thiobenzamide.

8-Br-cGMP did not affect the expression of either FMO1 mRNA or protein, whereas exposure to sper/NO resulted in a significant decrease of their expression (Figs. 5 and 6).

Discussion

We have shown that NO released from inflamed liver inhibits the activity of the major flavin-containing monooxygenase isoform in rat liver, FMO1, by reducing the steady-state mRNA stability in a cGMP-independent manner. This result indicates that post-transcriptional modification accounts for the NO-mediated decrease in FMO1 expression and activity under endotoxemic conditions.

The biochemical activity of FMOs, which metabolize endo- and exogenous compounds in several organs, including the liver, kidney, and brain, is suppressed in pathological human liver tissues, particularly by infections [2,3]. During this series of experiments, we reported that secondary fish-odor syndrome, also known as the metabolic disorder trimethylaminuria, which is caused by genetically defective FMO3 (a major isoform in human liver), can be acquired by NO-mediated impairment of hepatic FMO in hepatitis-B-virus-infected patients [7]. Moreover, we confirmed with an in vitro experiment that FMO3 activity is inhibited directly and reversibly by NO-mediated S-nitrosylation [6]. In a previous study, we reported that rat FMO1 mRNA expression and microsomal activity are suppressed under inflammatory conditions such as LPS-induced endotoxemia [5]. NO mediates this suppression, because the decrease in in vivo FMO1 mRNA expression was reversed completely by the addition of AG, a selective inhibitor of iNOS. At that time, however, it was unclear whether the effect of NO on mRNA suppression occurs in a transcriptional or post-transcriptional pathway.

Therefore, we examined the in vitro effects of NO donors in primary cultured rat hepatocytes on the actions of several putative transcription factors and on mRNA stability. However, NO had no inhibitory effect on the functions of the 5′-flanking regulatory sequences of the ~1.9-kb rat FMO1 gene, which contains putative binding sites for transcription factors such as HNF, C/EBP, JCV, and CF-1 (Ryu et al., unpublished data). At the

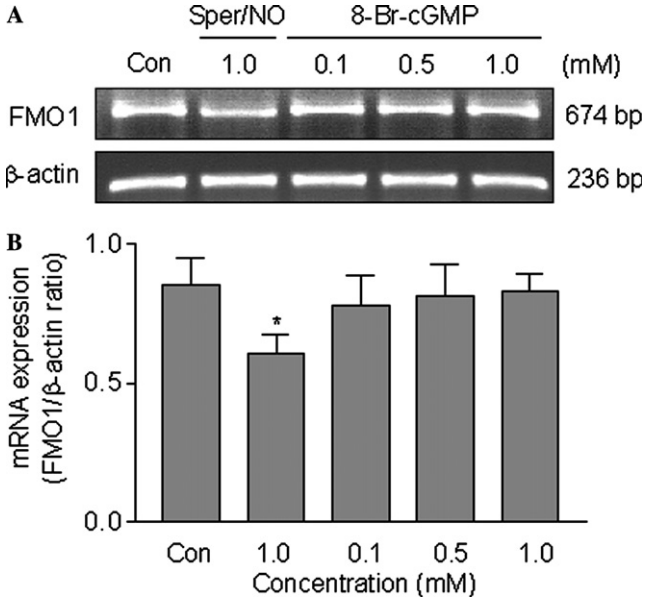

Fig. 5. Effects of 8-Br-cGMP on FMO1 mRNA levels in Act-D-untreated cells. (A) FMO1 mRNA levels were compared by RT-PCR in hepatocytes treated with 8-Br-cGMP at different concentrations and sper/NO at a single concentration. (B) In triplicate experiments, densitometric readings of FMO1 mRNAs normalized to β-actin bands are expressed as the ratio of FMO1/β-actin mRNA (*$p < 0.05$ vs controls, Student's t test).

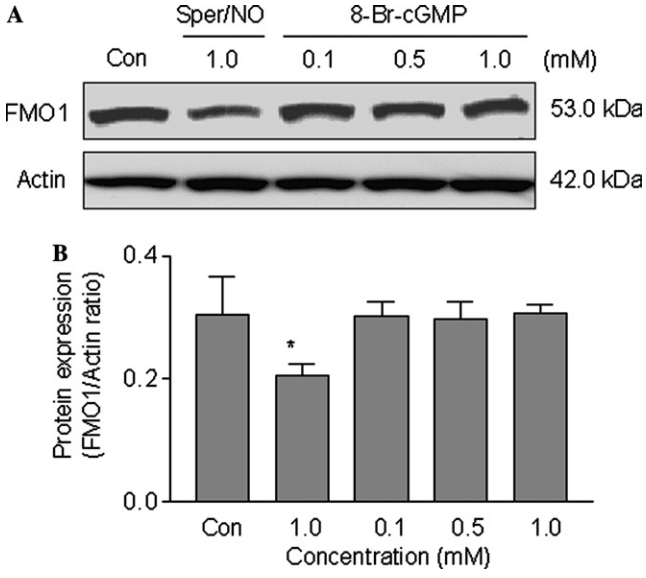

Fig. 6. Effects of 8-Br-cGMP on FMO1 protein levels in Act-D-untreated cells. (A) FMO1 protein levels were compared by Western blot analysis in hepatocytes treated with 8-Br-cGMP at different concentrations, and sper/NO at a single concentration. (B) In triplicate experiments, densitometric readings of FMO1 proteins normalized to actin bands are expressed as the ratio of FMO1/actin (*$p < 0.05$ vs controls, Student's t test).

same time, we tested another possibility by determining NO-mediated post-transcriptional modifications, like mRNA stability, in cells treated with the pure NO donor, spermine–NONOate, or in LCM-treated cells. These factors were used to investigate the effects of the endogenous NO released in cells, because iNOS is also induced in hepatocytes by stimulation with LPS or pro-inflammatory cytokines [11].

Because steady-state mRNA levels are regulated by the balance between its transcription and degradation, the mRNA stability maintained by the 7-methylguanosine cap structure at the 5′ end and the poly(A) tail at the 3′ end is an important factor determining transcript levels [26,27]. The mRNA decay rate is the major determinant in the mechanism controlling gene expression. The mRNA stability of many inducible genes encoding cytokines, lymphokines, and growth factors is controlled by RNA-stabilizing proteins (HuD, HuR, and AUF1) that bind to *cis*-acting elements, such as the AU-rich elements and CU- or U-rich elements in special motif sequences within the 3′ untranslated regions (3′-UTRs) [28–30]. The mRNA stability of these genes can be modified post-transcriptionally in response to extracellular or intracellular factors like cytokines and cytokine-induced free radicals.

Recently, several studies have shown that overproduced NO destabilizes the mRNAs of the modulated enzymes, like transforming growth factor β_3 [31], soluble guanylate cyclase [32], matrix metalloproteinase 9 [15,16], cytochrome *c* oxidase [17], and luciferase [33]. In contrast to the mRNA-destabilizing effects of NO, NO also stabilizes mRNAs of inducible enzymes such as oxidative-stress-mediated heme oxygenase 1 and TGF-β-inducible early response gene 1 [14,19,34]. These NO-dependent post-transcriptional mechanisms of gene regulation are important factors in gene expression [35]. Furthermore, as suggested by Filippov et al. [32], NO regulates gene transcription at the level of mRNA stability via cGMP-dependent or -independent mechanisms.

In general, FMO1 is expressed constitutively in the rat liver, but its activity or expression can be altered by hormones or some toxic chemicals [1,2]. Although there are no reports of FMO1 mRNA-stabilizing *cis*-acting elements or their binding proteins, the stability of FMO1 mRNA also seems to be a major target of NO. As shown in Fig. 2, because the transcriptional pathway is blocked completely in Act-D-treated cells, the decrease in FMO1 mRNA by NO after pretreatment with LCM is maintained at constant levels. However, in cells co-treated with Act-D and an NO donor, mRNA stability decreased dose-dependently, suggesting that NO is directly involved in this destabilization, with no involvement of cGMP, because gene transcription was blocked under these conditions (Fig. 3). In parallel experiments, treatment with the membrane permeable cGMP analog, 8-bromo-cGMP (0.1–1.0 mM), also did not affect FMO1 mRNA destabilization or FMO1 protein levels (Figs. 5 and 6). Especially in the presence of Act-D, treatment of cells with exogenous spermine–NONOate decreased FMO1 mRNA stability time- and dose-dependently,

resulting in a reduction in half-life (~280 min in 0.05 mM and ~60 min in 1 mM) induced by NO (Fig. 3). Treatment with selective inhibitors of guanylyl cyclase, ODQ (5 μM) or methylene blue (10 μM), also failed to block the destabilization FMO1 mRNA in NO-donor-treated cells, indicating the involvement of a cGMP-independent pathway (data not shown).

Interestingly, FMO1 mRNA (GenBank Accession No. BC061567) does not contain putative AU-rich elements, such as the AUUUA pentamer or U-rich elements, in the 3′-UTR, but contains three CUUUC pentamer sites similar to those identified in other gene mRNAs, such as that of myristoylated alanine-rich C kinase substrate (MARCKS) [30]. Therefore, we speculate that these sites may also be binding elements for mRNA-stabilizing ELAV/Hu proteins, although the detailed mechanism remains to be defined.

Our results demonstrate a novel mechanism of NO-mediated FMO1 mRNA destabilization in primary cultured rat hepatocytes, suggesting that overproduced NO accounts for the decreases in FMO activity and protein expression in inflamed livers. This may also be a pivotal mechanism leading to FMO-related liver dysfunctions, such as the acquired or secondary fish-odor syndrome observed in endotoxemic or many pathological conditions.

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